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Abstract: **BACKGROUND:** Approximately every third surgical patient is anemic. The most common form, iron deficiency anemia, results from persisting iron-deficient erythropoiesis (IDE). Zinc protoporphyrin (ZnPP) is a promising parameter for diagnosing IDE, hitherto requiring blood drawing and laboratory workup. **STUDY DESIGN AND METHODS:** Noninvasive ZnPP (ZnPP-NI) measurements are compared to ZnPP reference determination of the ZnPP/heme ratio by high-performance liquid chromatography (ZnPP-HPLC) and the analytical performance in detecting IDE is evaluated against traditional iron status parameters (ferritin, transferrin saturation [TSAT], soluble transferrin receptor-ferritin index [sTfR-F], soluble transferrin receptor [sTfR]), likewise measured in blood. The study was conducted at the University Hospitals of Frankfurt and Zurich. **RESULTS:** Limits of agreement between ZnPP-NI and ZnPP-HPLC measurements for 584 cardiac and noncardiac surgical patients equaled 19.7 mol/mol heme (95% confidence interval, 18.0-21.3; acceptance criteria, 23.2 mol/mol heme; absolute bias, 0 mol/mol heme). Analytical performance for detecting IDE (inferred from area under the curve receiver operating characteristics) of parameters measured in blood was: ZnPP-HPLC (0.95), sTfR (0.92), sTfR-F (0.89), TSAT (0.87), and ferritin (0.67). Noninvasively measured ZnPP-NI yielded results of 0.90. **CONCLUSION:** ZnPP-NI appears well suited for an initial IDE screening, informing on the state of erythropoiesis at the point of care without blood drawing and laboratory analysis. Comparison with a multiparameter IDE test revealed that ZnPP-NI values of 40 mol/mol heme or less allows exclusion of IDE, whereas for 65 mol/mol heme or greater, IDE is very likely if other causes of increased values are excluded. In these cases (77% of our patients) ZnPP-NI may suffice for a diagnosis, while values in between require analyses of additional iron status parameters.

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Screening for iron deficiency in surgical patients based on noninvasive zinc protoporphyrin measurements

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BACKGROUND: Approximately every third surgical patient is anemic. The most common form, iron deficiency anemia, results from persisting iron-deficient erythropoiesis (IDE). Zinc protoporphyrin (ZnPP) is a promising parameter for diagnosing IDE, hitherto requiring blood drawing and laboratory workup.

STUDY DESIGN AND METHODS: Noninvasive ZnPP (ZnPP-NI) measurements are compared to ZnPP reference determination of the ZnPP/heme ratio by high-performance liquid chromatography (ZnPP-HPLC) and the analytical performance in detecting IDE is evaluated against traditional iron status parameters (ferritin, transferrin saturation [TSAT], soluble transferrin receptor–ferritin index [sTfR-F], soluble transferrin receptor [sTfR]), likewise measured in blood. The study was conducted at the University Hospitals of Frankfurt and Zurich.

RESULTS: Limits of agreement between ZnPP-NI and ZnPP-HPLC measurements for 584 cardiac and noncardiac surgical patients equaled 19.7 $\mu\text{mol/mol}$ heme (95% confidence interval, 18.0–21.3; acceptance criteria, 23.2 $\mu\text{mol/mol}$ heme; absolute bias, 0 $\mu\text{mol/mol}$ heme). Analytical performance for detecting IDE (inferred from area under the curve receiver operating characteristics) of parameters measured in blood was: ZnPP-HPLC (0.95), sTfR (0.92), sTfR-F (0.89), TSAT (0.87), and ferritin (0.67). Noninvasively measured ZnPP-NI yielded results of 0.90.

CONCLUSION: ZnPP-NI appears well suited for an initial IDE screening, informing on the state of erythropoiesis at the point of care without blood drawing and laboratory analysis. Comparison with a multiparameter IDE test revealed that ZnPP-NI values of 40 $\mu\text{mol/mol}$ heme or less allows exclusion of IDE, whereas for 65 $\mu\text{mol/mol}$ heme or greater, IDE is very likely if other causes of increased values are excluded. In these cases (77% of our patients) ZnPP-NI may suffice for a diagnosis, while values in between require analyses of additional iron status parameters.

ABBREVIATIONS: ACD = anemia of chronic disease; AUC^{ROC} = area under the curve receiver operating characteristics; CRP = C-reactive protein; IDA = iron deficiency anemia; IDE = iron-deficient erythropoiesis; IL-6 = interleukin-6; LoAs = limits of agreement; MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; ROC = receiver operating characteristics; sTfR = soluble transferrin receptor; sTfR-F = soluble transferrin receptor–ferritin index; TSAT = transferrin saturation; ZnPP = zinc protoporphyrin; ZnPP-NI = noninvasive zinc protoporphyrin; ZnPP-HPLC = high-performance liquid chromatography zinc protoporphyrin.

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Iron is essential for an optimal erythropoiesis. Insufficiency results in iron-deficient erythropoiesis (IDE), which, if persistent, eventuates in iron deficiency anemia (IDA). In surgical patients, of which approximately one-third suffers from preoperative anemia, 23% to 72% of suboptimal hemoglobin (Hb) concentrations are associated with a shortage of erythropoietic iron supply.^{1–3} Preoperative anemia itself is an independent risk factor for a prolonged hospitalization, a higher need for allogeneic blood transfusions and increased morbidity and mortality.^{4–6} In iron-deficient patients, iron supplementation is a potent strategy to increase Hb levels and reduce transfusion requirements.^{7–10} Managing preoperative anemia by providing a pragmatic treatment within the often short time available to ensure optimal surgical preparation is an integral part of patient blood management, a multimodal clinical concept to improve patient safety.¹¹

Diagnosing anemia based on Hb levels is straightforward, yet only allows identification of patients already compromised by a reduced hemoglobin mass. Screening for IDE to provide treatment to patients already or not yet anemic due to iron deficiency, however, is much more challenging despite a plethora of iron status parameters. This is especially true when inflammation or comorbidities are present. A proxy highly sensitive for the iron availability during erythropoiesis is zinc protoporphyrin (ZnPP), primarily forming when iron supply to the erythron is insufficient or functionally impaired.^{12–17} In fact, ZnPP has been suggested to detect inadequate iron supply in different populations^{16,17} and children.¹⁵ Hitherto, however, ZnPP measurements required blood sampling and, to provide reliable results, additional washing of erythrocytes.^{18,19} A novel analytical technique sets out not only to render cumbersome laboratory workup obsolete²⁰ but also to enable the noninvasive quantification of ZnPP at the point of care without blood sampling and laboratory analysis.²¹

In this prospective multicenter study, we compare noninvasive ZnPP measurements to reference measurements and evaluate the analytical performance of the novel technique and of standard iron status parameters to detect IDE in a heterogeneous cohort of cardiac and noncardiac surgical patients.

MATERIALS AND METHODS

Study design and patients

This multicenter study was conducted at the University Hospitals of Frankfurt (UKF) and Zurich (USZ) and is registered as NCT03071497 (UKF) and NCT03231865 (USZ). Ethics committees at both sites approved the study protocol. Patients (aged ≥ 18 y) undergoing major elective cardiac and noncardiac surgery between March 2017 and April 2018 were included. Recent blood transfusion (< 8 wks before examination), pregnancy, or diagnosis of porphyria were

exclusion criteria. Written informed consent was obtained from each participant. Blood samples (UKF: 1 \times 2.6 mL ethylenediaminetetraacetic acid tube, 1 \times 4 mL serum tube; USZ: 1 \times 2.7 mL ethylenediaminetetraacetic acid tube, 1 \times 10 mL serum tube; both Sarstedt AG & Co. KG) for analyses were collected preoperatively during clinical routine.

Assessments

At both sites, two aliquots of ethylenediaminetetraacetic acid blood (250 μ L each) were stored at -80°C for reference determination of the ZnPP/heme ratio by high-performance liquid chromatography (ZnPP-HPLC), as previously described.²¹ Noninvasive ZnPP measurements (ZnPP-NI) were performed preoperatively at the study sites by various examiners with prototype devices (FIDscreen). Before study enrollment, the prototypes (one at each study site) were calibrated against an external optical standard, and the relative calibration factor between the two prototypes was fixed (for detailed description see Appendix S1 and Fig. S1, available as supporting information in the online version of this paper). The working principle of the prototypes is described elsewhere.²¹

At the UKF, red blood cell (RBC) indices (Hb, mean corpuscular hemoglobin [MCH], mean corpuscular volume [MCV]) were measured using an analyzer (XN-9000, Sysmex). An analyzer (Cobas 8000, Roche Diagnostics) was used to measure serum iron (module c701), serum ferritin (module e602, electrochemiluminescence immunoassay), serum transferrin (module c502), soluble transferrin receptor (sTfR; module c502, Tina-quant Soluble Transferrin Receptor assay), C-reactive protein (CRP; module c701, latex-enhanced immunoturbidimetry), and interleukin-6 (IL-6; e602, electrochemiluminescence immunoassay). At the USZ, RBC indices (Hb, MCH, MCV) were measured using an analyzer (XE-5000, Sysmex). The Cobas 8000 was used to measure serum iron (module c502), serum ferritin (module e801, electrochemiluminescence immunoassay), serum transferrin (module c502), CRP, and IL-6 (module e801). sTfR was measured using a nephelometric analyzer (Atellica Neph 630, Siemens Healthineers, immunonephelometry). Transferrin saturation (TSAT) was calculated as $\text{TSAT} = (\text{Serum iron } [\mu\text{g/dL}] / \text{Serum transferrin } [\text{mg/mL}]) \times 70.9$. sTfR-F index (sTfR-F) was calculated as $\text{sTfR-F} = \text{sTfR } [\text{mg/L}] / \log(\text{Serum ferritin}) [\text{ng/mL}]$.

Clinical definitions

Evaluating the analytical performance of iron status parameters to detect IDE requires an a priori identification of affected patients. Bone marrow aspiration, the gold standard to estimate the body iron stores, is highly invasive and not routinely performed at the study sites. As using a single iron status parameter for diagnosing IDE might result in an under- or overestimation of the incidence rate, a multiparameter index test was applied.^{22–24} Patients were assigned to the “IDE group” if at least two of three parameters (TSAT, sTfR, ZnPP-HPLC) indicated IDE, or the “no

IDE group" if none or only one parameter was positive. All three parameters were chosen for the initial identification, as they provide information on the availability of iron for erythropoiesis.^{14,25,26} Cutoff values of TSAT (<20%) and sTfR (women >4.4 mg/L, men >5 mg/L) are inferred from a consensus statement,²⁷ national guidelines,^{28,29} and recommendations by the manufacturer and central laboratories at both sites. The cutoff value of ZnPP (>40 $\mu\text{mol/mol}$ heme, applied for both ZnPP-HPLC and ZnPP-NI) is inferred from the literature,^{12,13,16,30} matching recommendations of the laboratory conducting the reference measurements. Cutoff values indicating reduced iron stores for ferritin (<100 ng/mL), MCH (<27 pg), and MCV (<80 fL) are inferred from recommendations by the central laboratories and the literature.²⁷ Although a lower ferritin cutoff (<30 ng/mL) is more specific for absolute iron deficiency,³¹ we preferred a higher cutoff (<100 ng/mL) suggested as indicative for low iron stores in surgical patients.²⁷ The reasoning here is that only replete iron stores (>100 ng/mL) ensure a supply sufficient for an optimal perioperative erythropoiesis even during major surgical blood loss. Furthermore, this cutoff was suggested as an indicator for iron deficiency in nonanemic patients with various comorbidities (e.g., chronic kidney disease, inflammatory bowel disease).³² Cutoff values of sTfR-F (women >2.2, men >2.5) were calculated as follows: women = $4.4 \text{ [mg/L]} / \log(100) \text{ [ng/mL]}$; men = $5 \text{ [mg/L]} / \log(100) \text{ [ng/mL]}$. To decide upon copresence of inflammation, we used CRP cutoff values greater than 5 mg/dL and/or IL-6 levels greater than 7 pg/mL, as recommended by the central laboratories (if both were available, CRP was preferred). Anemia, defined by the World Health Organization as Hb less than 12 g/dL in women and less than 13 g/dL in men, respectively,³³ was not used to divide the study population for further subgroup analyses, as IDE can be present irrespective of anemia. Nonetheless, as prevalence of IDA, anemia of chronic disease (ACD), a combination of both or other causes of anemia within the study groups ("no IDE" and "IDE") might be of interest for some readers, we used a modified version of an algorithm published by Skikne et al.³⁴ (for details see Fig. S2, available as supporting information in the online version of this paper) to provide these numbers within the Appendix S1.

Statistical analysis

Because all analyzed parameters were not normally distributed (Shapiro-Wilk-test; $p < 0.05$ considered significant), data are given as medians (interquartile range [IQR], Q1-Q3). Accordingly, differences between two groups were tested with the Mann-Whitney U test ($p < 0.05$ considered significant). To evaluate the analytical performance of iron status parameters to detect IDE, receiver operating characteristics (ROC) including area under the curve (AUC^{ROC}) and contingency tables were computed based on cutoffs listed in Clinical Definitions.

For direct comparison of noninvasive and reference ZnPP measurements (ZnPP-NI vs. ZnPP-HPLC), noninvasive values were scaled to the reference HPLC determination by a linear fit with zero offset (see Appendix S1 and Fig. S1, available as supporting information in the online version of this paper). For quantitative comparison of both methods, limits of agreement (LoAs) as well as absolute and relative biases were calculated using the robust τ -estimate of the differences between the two. The 95% confidence intervals (CIs) were calculated by bootstrapping. The LoAs were considered acceptable if they did not exceed the LoAs of repeated determinations of ZnPP by HPLC by more than 20%. All calculations were done with computer software (MATLAB R2014b, The MathWorks Inc.), with exception of the LoAs (95% CI), for which another software (R version 3.2.2, functions `scaleTau2`, `boot`, `boot.ci`) was used.

RESULTS

Initially, 649 patients undergoing major elective surgery were included, 65 of which were excluded because at least one measuring parameter was missing ($n = 44$), there were issues with the hardware or software of the noninvasive device ($n = 14$), the patient had received a blood transfusion less than 8 weeks before examination ($n = 6$), or for other reasons ($n = 1$; Fig. 1). The median age was 65 (IQR, 54–73; range, 18–91) years and 34.8% ($n = 203$) were female. Included patients were scheduled for cardiac ($n = 260$), general ($n = 39$), gynecologic ($n = 2$), hepatopancreatobiliary ($n = 68$), maxillofacial ($n = 3$), plastic ($n = 14$), rectal ($n = 15$), thoracic ($n = 7$), transplantation ($n = 43$), trauma ($n = 5$), upper gastrointestinal ($n = 40$), urological ($n = 25$), vascular ($n = 31$), or other ($n = 32$) surgery.

The LoAs between noninvasive and HPLC reference ZnPP measurements for $n = 584$ patients equaled 19.7 $\mu\text{mol/mol}$ heme (95% CI, 18.0–21.3) with no bias (Fig. 2). For comparison, LoAs between ZnPP values of repeated HPLC measurements were 19.3 $\mu\text{mol/mol}$ heme (95% CI, 17.8–20.6) for 583 patients with an absolute bias of $-2.8 \mu\text{mol/mol}$ heme and a relative bias of -7.3% (for Bland-Altman plot see Fig. S3, available as supporting information in the online version of this paper). Hence, LoAs between noninvasive and HPLC reference ZnPP measurements were within the predefined acceptance criteria ($23.2 \mu\text{mol/mol}$ heme = $1.2 \times 19.3 \mu\text{mol/mol}$ heme).

In total, 447 patients were assigned to the no IDE group (no [$n = 265$] or only one [$n = 182$] parameter indicating IDE; Fig. 1), in which medians of RBC indices, iron status parameters, and inflammation markers did not exceed or fall below respective cutoff values (Table 1). Twelve of those with only one parameter indicating IDE had ferritin values less than 30 ng/mL. In contrast, medians of RBC indices (except for MCH and MCV) and iron status parameters in the IDE group ($n = 137$), exceeded or were below cutoff

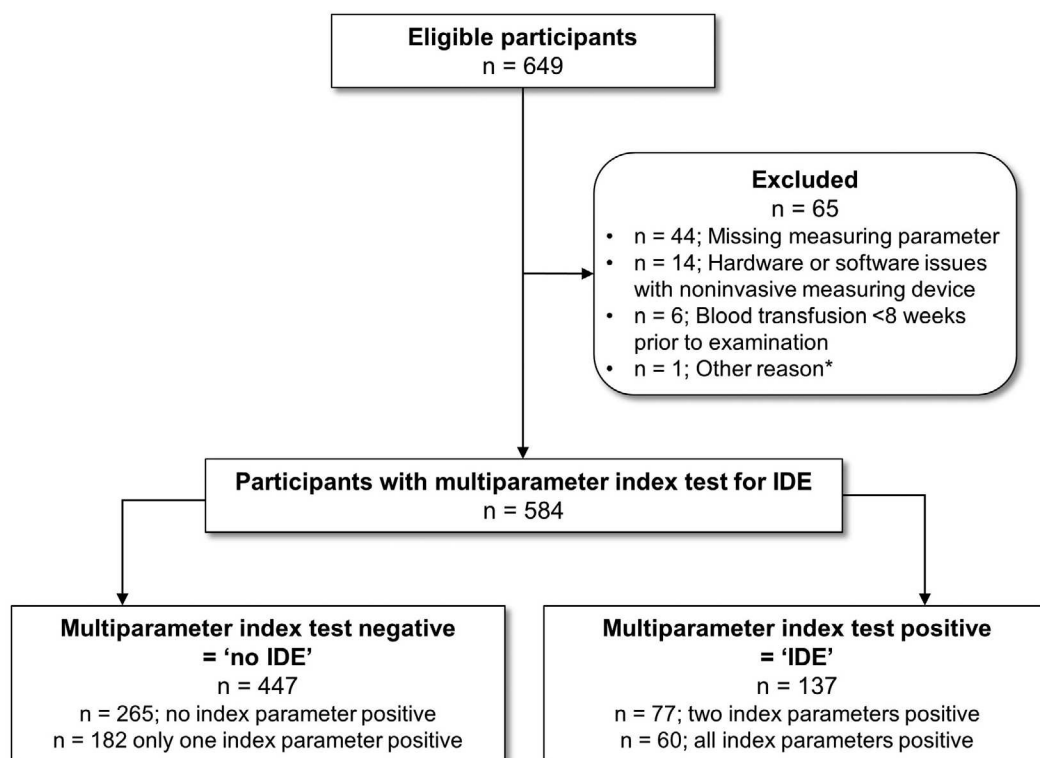


Fig. 1. Flowchart showing the study population. Parameters (and cutoffs) used for multiparameter index test to detect IDE: transferrin saturation (<20%), soluble transferrin receptor (>4.4 mg/L in women, >5 mg/L in men), and ZnPP measured with HPLC (>40 $\mu\text{mol/mol}$ heme). Patients were assigned to the no IDE group, if none or only one index parameter was positive or to the IDE group, if at least two index parameters were positive. IDE = iron-deficient erythropoiesis. *Blood sample likely contaminated, as it was taken through the same peripheral venous catheter previously used for intravenous iron supplementation.

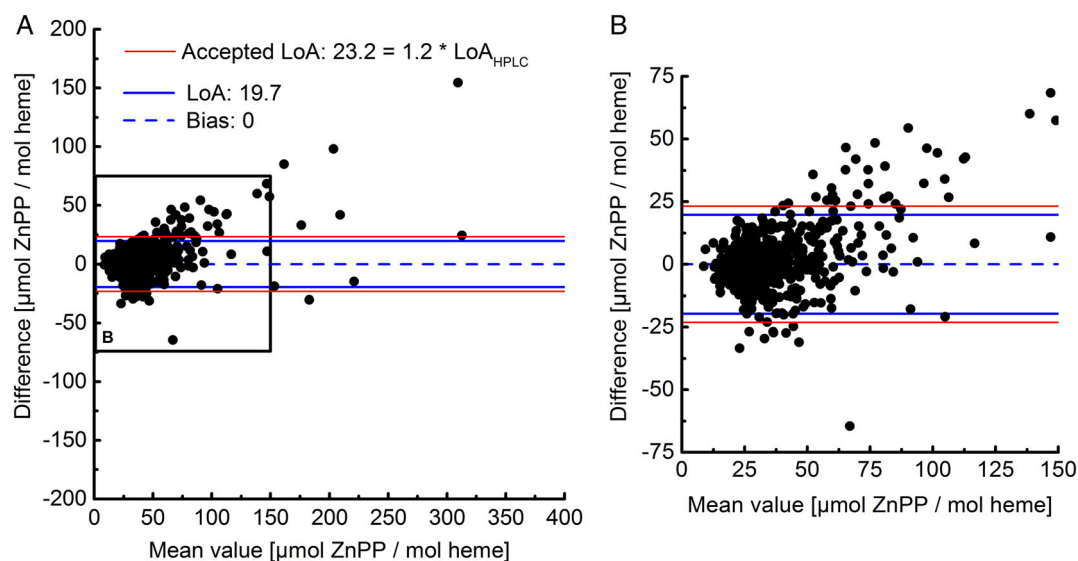


Fig. 2. Bland-Altman plots for the comparison of ZnPP measured in blood samples using high-performance liquid chromatography (ZnPP-HPLC) and noninvasively by fluorescence analysis (ZnPP-NI). (A) gives the complete data set, (B) depicts a magnification of (A). Solid blue lines indicate robust limits of agreement (equaling 1.96 times of the robust SD). Bias (equal to zero) is indicated by blue dashed line. Acceptance criteria are shown in red and are defined as 1.2 times the robust limits of agreement of repeated ZnPP determinations by HPLC.

TABLE 1. RBC indices, iron status parameters, and markers of inflammation for the no IDE group, the IDE group, and for all patients

Parameter	No IDE (n = 447)	IDE (n = 137)	p value	All (n = 584)
Hb (g/dL)	13.8 (12.6-14.8)	11.8 (10.6-13.1)	<0.0001	13.5 (12.0-14.6)
MCV (fL)	89.9 (86.7-93.4)	85.0 (81.5-89.8)	<0.0001	89.1 (85.3-92.6)
MCH (pg)	30.2 (29.1-31.3)	28.1 (25.7-29.5)	<0.0001	29.8 (28.5-31.0)
Ferritin (ng/mL)	173 (90.0-300.0)	93 (29-261)	<0.0001	159 (71-296)
TSAT (%)	24.0 (19.5-30.4)	12.1 (8.2-17.0)	<0.0001	21.9 (16.0-28.5)
sTfR-F	1.3 (1.0-1.6)	2.5 (1.9-3.8)	<0.0001	1.5 (1.1-2.1)
sTfR (mg/L)	2.8 (2.4-3.4)	5.1 (4.0-6.7)	<0.0001	3.1 (2.5-4.0)
ZnPP-HPLC (μmol/mol heme)	27.8 (21.6-34.6)	64.3 (47.7-88.1)	<0.0001	31.3 (23.2-45.3)
ZnPP-NI (μmol/mol heme)	29.6 (23.2-35.9)	51.9 (42.8-71.6)	<0.0001	33.1 (25.2-44.2)
CRP (mg/dL)	3.0 (1.0-7.0)	7.9 (2.0-18.4)	<0.0001	3.0 (1.0-8.9)
IL-6 (pg/mL)	3.6 (2.0-7.2)	5.8 (3.5-9.6)	0.044	4.2 (2.1-7.3)

Results are median (IQR). Mann–Whitney U tests comparing parameters between the group of patients with normal (no IDE) and impaired erythropoiesis (IDE) revealed significant differences.

CRP = C-reactive protein; Hb = hemoglobin; IDE = iron-deficient erythropoiesis; IL-6 = interleukin-6; MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; sTfR = soluble transferrin receptor; sTfR-F = soluble transferrin receptor–F index; TSAT = transferrin saturation; ZnPP-HPLC = high-performance liquid chromatography zinc protoporphyrin; ZnPP-NI = noninvasive zinc protoporphyrin.

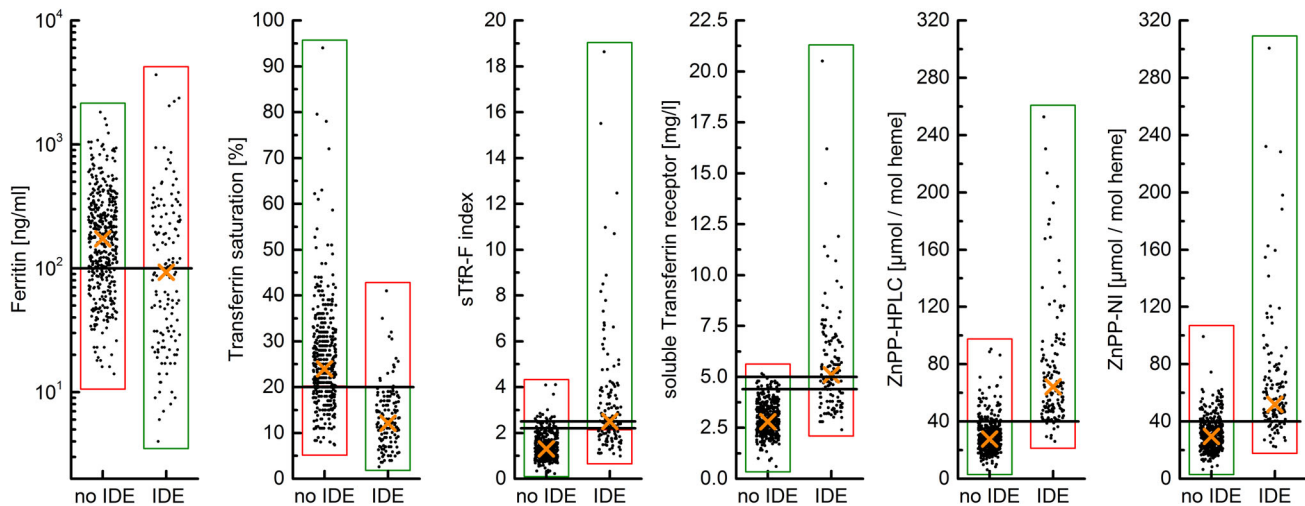


Fig. 3. Distribution of ferritin, TSAT, sTfR-F, sTfR, ZnPP-HPLC, and ZnPP-NI values in patients with normal (no IDE, n = 447) and iron-deficient (IDE, n = 137) erythropoiesis. Black horizontal lines depict respective iron deficiency cutoff value of each parameter. For sTfR-F and sTfR, the upper line represents the cutoff for men, the lower one the cutoff for women. Medians are marked by orange crosses. Colored rectangles envelope patients correctly (green) or falsely (red) assigned to no IDE/IDE group by respective parameters. Note: ferritin is shown on a logarithmic scale.

values, consistently indicating iron deficiency and IDE, respectively. The median of CRP (7.9 mg/dL; IQR, 2.0–18.4) also exceeded cutoff, indicating a higher prevalence of inflammation in this group. Ferritin values of both groups showed considerable overlap, whereas values of sTfR, ZnPP-HPLC, and ZnPP-NI allowed a slightly better visual separation (Fig. 3). For results of additional screening for anemia (and potential causes) within the study groups based on a modified version of an algorithm published elsewhere,³⁴ see Table S1, available as supporting information in the online version of this paper.

Iron status parameters differed markedly regarding their analytical performance to detect IDE (Table 2). For the

total population, overall analytical performance (inferred from AUC^{ROC}) declined from ZnPP-HPLC (0.95), to sTfR (0.92), to ZnPP-NI (0.90), to sTfR-F (0.89), to TSAT (0.87), and to ferritin (0.63; for ROC curves see Fig. S4, available as supporting information in the online version of this paper). Based on predefined cutoffs (see Clinical Definitions), ferritin revealed lowest sensitivity (51%) and specificity (72%). Highest specificities were achieved by sTfR (99%) and sTfR-F (95%), with both accompanied by low sensitivities (61% for both). Highest equally balanced sensitivity (94%) and specificity (87%) were achieved by ZnPP-HPLC. ZnPP-NI likewise revealed balanced values for sensitivity (81%) and specificity (84%). Computed optimal cutoff values for the

TABLE 2. Analytical performance of iron status parameters to detect patients with IDE depending on the probability of copresence of inflammation

	Ferritin	TSAT	sTfR-F	sTfR	ZnPP-HPLC	ZnPP-NI
All patients (n = 584)						
Sens	51%	89%	61%	61%	94%	81%
Spec	72%	73%	95%	99%	87%	84%
PPV	36%	51%	80%	94%	69%	61%
NPV	83%	96%	89%	89%	98%	94%
False neg	67	15	54	54	8	26
False pos	126	119	21	5	58	72
True neg	321	328	426	442	389	375
True pos	70	122	83	83	219	111
AUC ^{ROC}	0.63	0.88	0.89	0.92	0.95	0.90
Cutoff	101 ng/mL	19.0%	1.7	3.8 mg/L	40.0 µmol/mol heme	39.6 µmol/mol heme
Patients without inflammation (n = 359)						
Sens	59%	84%	68%	60%	95%	83%
Spec	70%	79%	96%	99%	88%	88%
PPV	30%	46%	77%	95%	63%	59%
NPV	89%	96%	93%	92%	99%	96%
False neg	26	10	20	25	3	11
False pos	88	62	13	2	35	36
True neg	208	234	283	294	261	260
True pos	37	53	43	38	60	52
AUC ^{ROC}	0.68	0.87	0.90	0.92	0.96	0.91
Cutoff	85 ng/mL	19.8%	2.1	3.4 mg/L	40.2 µmol/mol heme	39.4 µmol/mol heme
Patients with inflammation (n = 225)						
Sens	45%	93%	54%	61%	93%	80%
Spec	75%	62%	95%	98%	85%	76%
PPV	46%	55%	83%	94%	75%	62%
NPV	73%	95%	81%	84%	96%	88%
False neg	41	5	34	29	5	15
False pos	38	57	8	3	23	36
True neg	113	94	143	148	128	115
True pos	33	69	40	45	69	59
AUC ^{ROC}	0.61	0.86	0.88	0.92	0.94	0.86
Cutoff	122 ng/mL	17.1%	1.8	4.0 mg/L	40.0 µmol/mol heme	40.1 µmol/mol heme

Cutoff values used to compute sensitivity (sens), specificity (spec), positive predictive value (PPV), and negative predictive value (NPV) were <100 ng/mL for ferritin, <20% for TSAT, >4.4 mg/L for women and >5 mg/L for men for sTfR, >2.2 for women and >2.5 for men for sTfR-F, and >40 µmol/mol heme for ZnPP-HPLC and ZnPP-NI. Cutoffs for the patient group without inflammation were CRP ≤5 mg/dL and IL-6 ≤7 pg/mL, respectively. Cutoff values displayed in this table were computed using ROC analyses.

AUC^{ROC} = area under the curve receiver operating characteristics; IL-6 = interleukin-6; ROC = receiver operating characteristics; sTfR = soluble transferrin receptor; sTfR-F = soluble transferrin receptor-F index; TSAT = transferrin saturation; ZnPP-HPLC = high-performance liquid chromatography zinc protoporphyrin; ZnPP-NI = noninvasive zinc protoporphyrin.

total study population of ferritin (101 ng/mL), TSAT (19.0%), ZnPP-HPLC (40.0 µmol/mol heme), and ZnPP-NI (39.6 µmol/mol heme) matched conventional reference limits, while those computed for sTfR-F (1.7) and sTfR (3.7 mg/L) remained below. For analytical performance based on the computed optimal cutoffs see Table S2, available as supporting information in the online version of this paper.

To evaluate potential effects of inflammation on iron status parameters, the study population was separated into four subgroups, based on sTfR levels and probability of inflammation inferred from CRP and IL-6 (Table 3). sTfR was explicitly chosen as differentiator for the state of erythropoiesis, as it is suggested to be the parameter least influenced by inflammation.^{23,34,35} Corroborating these findings, medians of sTfR in the group with normal sTfR differed only slightly between subgroups with (3.0 mg/L) and without inflammation (2.9 mg/L). For ZnPP-HPLC and

ZnPP-NI, medians were higher in the subgroup with (31.6 and 34.7 µmol/mol heme) compared to the subgroup without inflammation (28.3 and 29.6 µmol/mol heme), although remaining within normal range (≤40 µmol/mol heme). Ferritin and TSAT displayed large differences, with ferritin levels being higher (201 ng/mL vs. 150 ng/mL) and TSAT being lower (20.0% vs. 24.1%) in the subgroup with inflammation. In the group with elevated sTfR, ferritin was again higher by approximately 50 ng/mL in the subgroup with inflammation (97 ng/mL vs. 49 ng/mL), whereas TSAT was again lower in this subgroup (10.8% vs. 11.7%). In the subgroup with inflammation, sTfR, ZnPP-HPLC, and ZnPP-NI were lower compared to the subgroup without. Aside from sTfR-F, differences between medians of iron status parameters in the larger group with sTfR normal (n = 496) were significant, whereas those in the smaller group with sTfR elevated (n = 88) were not. Additionally, for all iron status parameters besides sTfR, a smaller AUC^{ROC} for the detection

TABLE 3. Iron status parameters in dependence on probability of inflammation

Parameter	sTfR normal (n = 496)			sTfR elevated (n = 88)		
	No inflammation (n = 319)	Inflammation (n = 177)	p value	No inflammation (n = 40)	Inflammation (n = 48)	p value
Ferritin (ng/mL)	150 (79-266)	201 (94-358)	0.0034	49 (21-198)	97 (31-321)	0.1
TSAT (%)	24.1 (19.2-30.2)	20.0 (15.0-28.0)	<0.0001	11.7 (7.1-21.8)	10.8 (7.0-16.8)	0.38
sTfR-F	1.4 (1.0-1.7)	1.3 (1.0-1.8)	0.67	3.4 (2.6-5.6)	2.9 (2.2-4.0)	0.09
sTfR (mg/L)	2.9 (2.4-3.4)	3.0 (2.5-3.8)	0.02	5.9 (5.4-7.3)	5.7 (5.0-7.3)	0.44
ZnPP-HPLC (μmol/mol heme)	28.3 (21.5-36.3)	31.6 (23.9-42.6)	0.0011	69.5 (49.7-96.6)	65.1 (46.6-97.9)	0.80
ZnPP-NI (μmol/mol heme)	29.6 (23.3-36.1)	34.7 (26.1-45.0)	0.0004	57.7 (46.2-74.1)	50.4 (39.9-84.4)	0.66
CRP (mg/dL)	1.0 (1.0-2.2)	10.5 (6.5-24.0)	<0.0001	1.7 (1.0-2.5)	12.6 (9.5-34.9)	<0.0001
IL-6 (pg/mL)	3.0 (1.5-4.1)	12.9 (8.7-16.5)	<0.0001	5.2 (3.1-6.0)	14.0 (8.2-18.0)	0.0003

Cutoffs for "sTfR normal" were ≤ 4.4 mg/L in women and ≤ 5 mg/L in men. Cutoffs for "no inflammation" were CRP ≤ 5 mg/dL and IL-6 ≤ 7 pg/mL, respectively. Results are median (IQR). In the patient group with normal sTfR values, Mann-Whitney U tests revealed significant differences in the medians of all parameters, except the sTfR-F index, between the subgroup of patients with and without inflammation. In the group of patients with elevated sTfR values, there were no statistical differences for the medians of all parameters, except for CRP and IL-6, between the subgroup of patients with and without inflammation.

CRP = C-reactive protein; IL-6 = interleukin-6; sTfR = soluble transferrin receptor; sTfR-F = soluble transferrin receptor-F index; TSAT = transferrin saturation; ZnPP-HPLC = high-performance liquid chromatography zinc protoporphyrin; ZnPP-NI = noninvasive zinc protoporphyrin.

of IDE is found in the group of patients with inflammation compared to the group without (Table 2).

DISCUSSION

In many surgical patients, anemia is associated with an impaired erythropoiesis due to iron deficiency,¹⁻³ thus readily treatable if timely diagnosed.⁷⁻¹⁰ Data of this prospective multicenter study including 584 cardiac and noncardiac surgical patients demonstrate that a novel noninvasive technique to measure ZnPP allows detecting IDE with higher analytical performance compared to the frequently used iron status parameters ferritin and TSAT.

Of all investigated parameters, ferritin showed the poorest analytical performance in detecting IDE, for which there are likely several reasons. First, ferritin is not sensitive to IDE, as it reflects body iron stores. Hence, values below cutoff do not necessarily indicate IDE, as iron supply might still be adequate (e.g., through phagocytosis of old erythrocytes), potentially explaining the high number of 126 false-positive results for IDE. Second, due to its acute-phase reactant properties and the high number of patients with copresence of unspecific inflammation (39%), ferritin values might be falsely "normal" in many cases, potentially explaining the high number of 67 false-negative results for IDE. Nevertheless, ROC analysis of ferritin versus IDE yielded a cutoff of 101 ng/mL, matching the suggested cutoff (100 ng/mL) for this cohort.²⁷ Calculations based on a lower cutoff (<30 ng/mL) frequently suggested as most sensitive and specific test to diagnose absolute iron deficiency,³¹ resulted in a decrease of false-positive results ($n = 12$), while false negatives in turn increased ($n = 102$). The resulting very high specificity (97%) and very low sensitivity (26%) for detecting IDE are likely also caused by the reasons stated above. Although TSAT clearly outperformed ferritin in detecting IDE, many patients were still misclassified. In simplified terms, TSAT is the ratio of iron to

transferrin in serum. With the former sensitive to prolonged overnight fasting and diurnal variations³⁶ and the latter being an anti-acute-phase protein,³⁷ both may affect TSAT values. Thus, although ferritin and TSAT are frequently recommended when screening for iron deficiency,²⁷⁻²⁹ diagnostic results might be affected by concomitant inflammation. A potential candidate to track erythropoietic iron demand independent from inflammation is sTfR.^{23,34,35} This is supported by a good analytical performance even in the presence of inflammation observed here ($AUC^{ROC} = 0.92$ in patients with and without inflammation). The low sensitivity (61%) likely resulted from the diverging cutoffs recommended by the manufacturer (>4.4 mg/L women, >5 mg/L men) and optimal values computed by ROC analysis for the studied population (3.8 mg/L). Computing sTfR-F is suggested to further improve the analytical sensitivity,^{34,35} which was not observed here, however, likely due to the high number of ferritin values affected by inflammation. Despite good analytical results, hospitals may hesitate with the routine use of sTfR due to analytical costs (e.g., UKF = 14.57 €; USZ = 77.43 CHF) or different reference ranges of commercially available reagents.²⁶

Another proxy for iron availability during erythropoiesis is ZnPP, primarily forming when iron supply to the erythron is insufficient or functionally impaired and divalent zinc instead of ferrous iron is incorporated into the heme scaffolding.¹²⁻¹⁷ With changes in ZnPP becoming visible with the formation of new and eryptosis of old erythrocytes, ZnPP provides long-term information (approx. 3 months) on the iron status—a fact that needs consideration when screening patients who recently received allogeneic blood, as donor ZnPP levels might affect measured values. Data presented here support the suggested good analytical performance of ZnPP to detect IDE, with ZnPP-HPLC yielding results at least equaling analytical performance of sTfR in the entire study population (AUC^{ROC} ZnPP-HPLC = 0.95) as well as within the subgroup of patients suffering from inflammation (AUC^{ROC} ZnPP-HPLC = 0.94). Hitherto, ZnPP

is rarely found in clinical routines, potentially because results of the most prevalent analytical technique (front-face fluorometry on a drop of blood) can be falsely increased by simultaneously detecting background signals of other fluorophores (e.g., bilirubin) if blood samples are measured unwashed.^{18,19} A novel analytical technique circumvents these issues by means of dual-wavelength excitation allowing the noninvasive quantification of ZnPP at the point of care without extensive laboratory equipment or blood sampling.^{20,21} An optical fiber probe illuminates the patient's lower lip and acquires fluorescence emission intensity of erythrocyte bound ZnPP, based on which information on the erythropoietic iron supply is available within minutes.²¹ Corroborating previous findings, the LoAs between ZnPP-HPLC reference measurements in blood and ZnPP-NI in the more heterogeneous collective studied here (19.7 $\mu\text{mol/mol}$ heme) equaled results of the group of postpartum women of a previous study (19.0 $\mu\text{mol/mol}$ heme).²¹ Analytical performance of ZnPP-NI ($\text{AUC}^{\text{ROC}} = 0.90$) in detecting IDE compared well to standard iron status parameters measured in blood (AUC^{ROC} of ZnPP-HPLC = 0.95; sTfR = 0.92; sTfR-F = 0.89; TSAT = 0.87; ferritin = 0.62). When analysis was focused on patients with high probability of inflammation, analytical performance of ZnPP-NI to detect IDE remained good ($\text{AUC}^{\text{ROC}} = 0.86$). However, in iron-replete patients (based on sTfR), ZnPP-NI values were increased in the subgroup with higher probability of inflammation (34.7 vs. 29.6 $\mu\text{mol/mol}$ heme, $p < 0.05$; Table 3). This might partly be explained by ZnPP not only being increased by absolute iron deficiency but also by impaired iron bioavailability during ACD, including chronic inflammation.¹³ With differences between both subgroups being relatively small and both medians remaining below cutoff, however, a major impact of chronic inflammatory disorders on the measured ZnPP-NI in the studied cohort seems rather unlikely. In iron-replete patients, raised ZnPP values can also result from lead exposure or genetic/acquired disorders including myelodysplastic syndrome,³⁰ α - and β -thalassemia traits,³⁸ hemoglobinopathies, porphyrias, and sideroblastic/inherited microcytic anemias,²¹ with strongly varying prevalence depending on the patient group in focus. When these diagnoses are known, other, less affected iron status parameters should be consulted. Especially in populations with patients from Africa, the Mediterranean, and Southeast Asia, mild and undiagnosed hemoglobinopathies should be considered.

To minimize risk of potential misdiagnosis due to slightly elevated values when using ZnPP-NI as initial screening parameter for IDE (e.g., as part of preoperative anemia management programs) we suggest a transition zone, in which ZnPP-NI diagnoses are largely indefinite and should thus be verified by other parameters (TSAT and sTfR). The lower limit of this transition zone (40 $\mu\text{mol/mol}$ heme) represents the mean cutoff calculated by ROC analysis (corresponding well with the literature^{12,13,16,30}). A reasonable definition of the upper limit is 65 $\mu\text{mol/mol}$ heme,

including more than 95% of false-positive identified patients in the transition zone. Values outside of the transition zone are either indicative for IDE unlikely (≤ 40 $\mu\text{mol/mol}$ heme) or IDE likely (≥ 65 $\mu\text{mol/mol}$ heme). When putting this algorithm to a test in the heterogeneous study population, diagnoses based on noninvasive ZnPP values outside the transition zone were largely corroborated by results of the multiparameter index test (Fig. 4). In fact, of the 401 patients with ZnPP-NI of 40 $\mu\text{mol/mol}$ heme or less, 94% ($n = 375$) were likewise diagnosed as not suffering from IDE by the multiparameter index test (i.e., part of the "no IDE" group), whereas for the 47 patients with ZnPP-NI of 65 $\mu\text{mol/mol}$ heme or greater, 96% ($n = 45$) were likewise classified as suffering from IDE (IDE group). That implies that in 77% ($n = 448$) of the study population, additional blood drawings and laboratory work to diagnose IDE might have been unnecessary, as largely coinciding results can be achieved noninvasively, quicker, and at the point of care. ZnPP-NI values in the transition zone (>40 and <65 $\mu\text{mol/mol}$ heme, $n = 136$; 23%) were clearly indefinite regarding IDE (no IDE, $n = 70$; 51% vs. IDE, $n = 66$; 49%, following multiparameter test) and should thus be verified by other parameters (TSAT and sTfR). We furthermore emphasize that when treatment is provided for IDE diagnosed by ZnPP-NI, potential confounding factors must be excluded.

This study has some limitations that should be mentioned. First, patients who received iron supplementation less than 8 weeks before examination were not excluded. With iron status parameters naturally changing following iron supplementation and some responding earlier than others, this might have affected the presented results. With the low number of patients potentially affected by intravenous iron supplementation (UKF = 6; USZ = 16), the impact on the overall cohort is presumably small. Second, no details on comorbidities or chronic underlying diseases besides inflammation are provided. Third, no in-depth differential diagnosis of patients suffering from IDA or ACD was performed for the study population, which is part of an optimal patient blood management. Fourth, the suggested IDE screening algorithm was tested on the same population used for its compilation and not on an independent validation cohort. Hence, future studies should test if the cutoffs suggested in the algorithm remain valid in other populations.

In conclusion, ZnPP-NI measurements allowed for detecting IDE in a large cohort of surgical patients. Analytical performance with and without concomitant inflammation was higher compared to the frequently used parameters ferritin and TSAT measured in blood. Contrasting traditional parameters, the novel technique provides point of care information on the erythropoiesis within minutes without the need for blood sampling and laboratory analyses. An additional advantage of the new technique is the low associated costs per measurement, as only a disposable plastic cover employed for hygienic reasons with manufacturing costs less than US\$1 is needed, but no

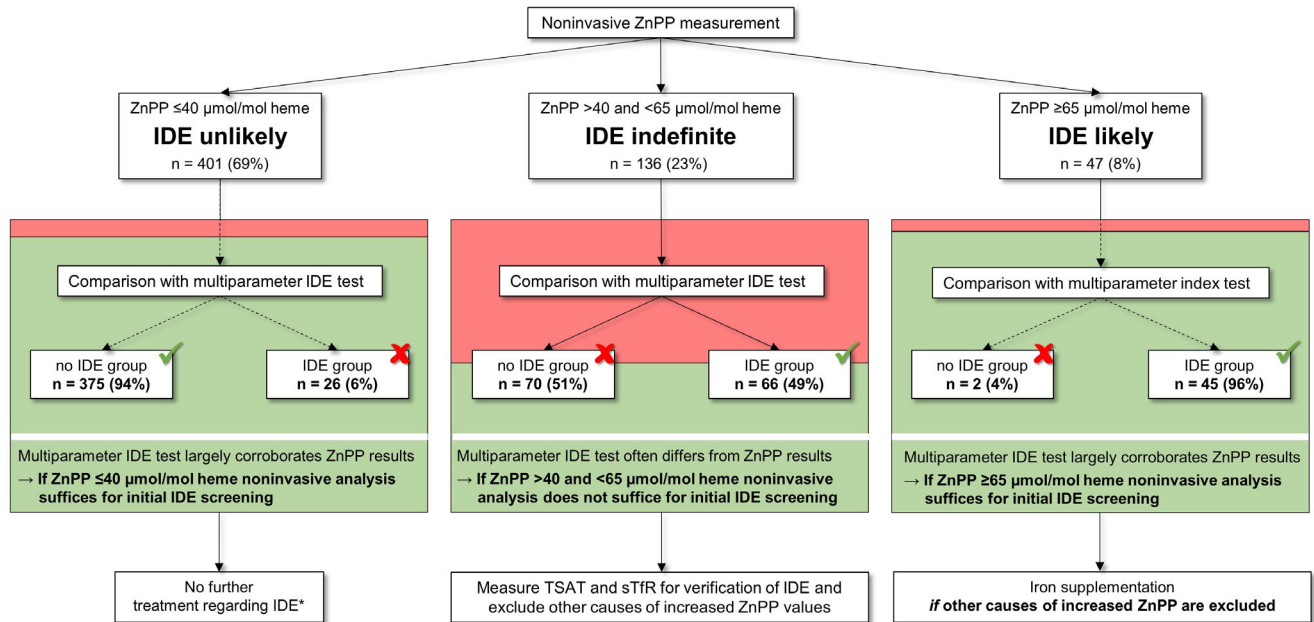


Fig. 4. Algorithm for an initial screening of IDE in surgical patients based on ZnPP-NI measurements. For ZnPP-NI values ≤ 40 $\mu\text{mol/mol}$ heme IDE is unlikely, whereas for values ≥ 65 $\mu\text{mol/mol}$ heme IDE is likely. ZnPP-NI values in the transition zone (>40 and <65 $\mu\text{mol/mol}$ heme) are indefinite and should be verified by measuring TSAT and sTfR. Diagnoses based on ZnPP-NI are compared to those of multiparameter IDE tests (minimum two of three parameters measured in blood [TSAT, sTfR, and ZnPP-HPLC] positive indicate IDE). Consent is indicated by green tick, dissent by red cross. Large rectangles are colored depending on the ratio of consents (green area) to dissents (red area) within each of the three regimes (IDE unlikely, IDE indefinite, and IDE likely). * marks settings in which analysis of ferritin could be helpful in identifying patients with iron depletion, that is, empty iron stores.

chemicals or biologic assays. Hence, if other causes of increased values either have a low prevalence in the population in focus or can be excluded (e.g., via patient history), ZnPP-NI measurements seem well suited as an initial screening parameter for IDE. Potential fields of application might be the routine screening of surgical patients as part of patient blood management programs, out-of-hospital medical offices, or resource-limited settings.

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performed all statistical analyses. GHi realized blood measurements at the UKF.


CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Supplementary material.